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Reduction in platelet-derived growth factor receptor mRNA in v-*src*-transformed fibroblasts

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Abstract

The status of the platelet-derived growth factor (PDGF) receptor in normal rat kidney (NRK) fibroblasts and in NRK fibroblasts transformed by the v-*src* oncogene or the polyoma middle T (pmt) antigen has been compared. v-*src*-NRK cells have 7-fold fewer surface binding sites for PDGF than NRK cells, but the affinity of the residual receptors for PDGF is reduced only 2-fold. Levels of the PDGF receptor measured by Western blotting or in an autophosphorylation assay in vitro are 8- and 4-fold lower respectively in v-*src*-NRK cells than in NRK cells. No PDGF-induced phosphorylation of the PDGF receptor is apparent after ^{32}P-labelling of intact v-*src*-NRK cells, implying that the reduction in PDGF receptor levels is not a consequence of production of autocrine PDGF. A 10-fold reduction in the amount of mRNA for the PDGF receptor is also observed in v-*src*-NRK cells. No decrease in PDGF receptor protein or mRNA levels is observed in pmt-NRK cells. We conclude that levels of the PDGF receptor in v-*src*-transformed NRK fibroblasts are modulated by reduction in the level of PDGF receptor mRNA.

Keywords: Platelet-derived growth factor; Receptor; mRNA; v-*src* Transformation; (Rat kidney fibroblast)

1. Introduction

Transformation by viral oncogenes frequently results in a profound reduction in the binding of growth factors to their receptors on the cell surface [1,2]. Two common mechanisms by which these effects are achieved are a reduction in receptor mRNA [3], or autocrine production of growth factor [1,2]. In the latter case internalization of occupied receptor leads to subsequent intracellular degradation, and hence to a reduction in the number of cell surface binding sites.

Down-regulation of EGF receptor mRNA has been observed previously following transformation by the v-*src* oncogene. Infection of normal rat kidney (NRK) [4] or Rat-1 [5] fibroblasts with a retrovirus carrying a temperature-sensitive v-*src* oncogene resulted in a greater than 90% reduction in the numbers of EGF receptors at the permissive temperature, with no apparent change in receptor affinity [4]. Although other workers have reported that mouse 3T3 fibroblasts transformed by a temperature-sensitive v-*src* oncogene showed a 10-fold reduction in EGF receptor affinity at the permissive temperature, with no apparent change in receptor number [6], Northern blotting experiments were consistent with a reduction in EGF receptor levels since a significant reduction in the amounts of EGF receptor mRNA was observed following v-*src* induction [3]. Modulation of the PDGF receptor at the RNA level following v-*src* transformation has not been reported previously.

Autocrine growth factor production has been observed in many fibroblast cell lines generated by transformation with viral oncogenes other than v-*src*. Secretion of transforming growth factor-α (TGF-α), an EGF homologue which binds to the EGF receptor, resulted in increased receptor internalization and degradation [1]. Fibroblasts transformed by SV40 virus, adenovirus, Kirsten and Moloney murine sarcoma viruses or simian sarcoma virus secreted a PDGF-like molecule into the medium with a resultant 50–100% decrease in PDGF receptor levels [2].

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NRK, normal rat kidney; PDGF, platelet-derived growth factor.

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Although the v-*src* oncogene was not tested in these experiments [2], Weber and co-workers observed no alteration in binding of PDGF to v-*src*-transformed Rat-1 fibroblasts [3]. The observation that PDGF-induced c-*fos* expression in v-*src*-transformed mouse 3T3 fibroblasts was still 60% of the level observed in untransformed cells [7] also suggested that v-*src* transformation did not reduce PDGF receptor levels by more than 40%.

A third distinct mechanism for down-regulation of PDGF receptor signalling was observed in mouse fibroblasts transformed by the v-*ras* oncogene. These cells expressed normal levels of PDGF receptor as measured by Western blotting [8], or by binding of iodinated PDGF [9], but PDGF-dependent receptor autophosphorylation was profoundly suppressed by an inhibitory factor present in the cell membranes [8] with a consequent attenuation in downstream signalling [9,10].

During a study of the effects of PDGF on the association between phosphatidylinositol 3-kinase and the PDGF receptor, we noticed that PDGF-dependent autophosphorylation of the PDGF receptor was profoundly suppressed in v-*src*-transformed NRK fibroblasts [11]. We therefore investigated the levels of PDGF receptor protein and its associated tyrosine kinase activity in these cells, and in NRK fibroblasts transformed by the polyoma virus middle T (pmt) antigen. This analysis was restricted to the β-type PDGF receptor since the major mitogenic effect of PDGF in human fibroblasts is mediated via the β-type receptor [12]. We report here that v-*src* transformation of NRK fibroblasts results in a reduction in the levels of PDGF receptor mRNA, with a consequent reduction in the amounts of PDGF receptor at the cell surface. Interestingly, PDGF receptor levels are not changed following pmt transformation.

2. Materials and methods

2.1. Materials

PDGF (BB homodimer) and rabbit anti-PDGF receptor (β type) polyclonal antiserum (PDGFR-3 [13]) were generous gifts from A. Thomasen (Amgen, Thousand Oaks, CA) and C.-H. Heldin (Ludwig Insitute, Uppsala, Sweden) respectively. ^{125}I-Bolton and Hunter-labelled PDGF BB homodimer (1.13×10^6 cpm/pmol) was purchased from Amersham, Bucks., UK. γ-[^{32}P]ATP and [^{32}P]orthophosphate were from Bresatec, Adelaide, South Australia. Normal rat kidney fibroblasts (NRK 49 F) were from the American Type Culture Collection, Rockville, MD. The derivation by Bruce Mann (Ludwig Institute, Melbourne, Australia) of stable NRK transformants expressing either the pmt or the v-*src* oncogene, and the evidence that the proteins encoded by both oncogenes are active, has been presented previously [11]. Unless otherwise indicated v-*src*-NRK clone 1 was used for all experiments.

2.2. Methods

Binding assay. The protocol for measurement of PDGF receptor binding has been described in detail previously [14]. Briefly, cells were grown to confluence in 24-well plates, and serum starved overnight prior to assay. For duplicate binding assays, cells were incubated for 30 min at 37°C in 0.5 ml 1% bovine serum albumin in Dulbecco's modified Eagle's medium containing a fixed concentration of ^{125}I-PDGF (50 pM) and increasing concentrations of unlabelled PDGF (3 pM–800 pM). Non-specific binding was determined by addition of a 100-fold excess of unlabelled PDGF. Cells were rinsed twice with phosphate-buffered saline, lysed in 1 M NaOH, and transferred to γ-counter tubes for counting of bound ^{125}I-PDGF. Cell numbers in a duplicate well were determined by counting in a hemocytometer after trypsinization.

In vitro kinase assay. Confluent cells were placed in Dulbecco's modified Eagle's medium without serum for 20 h, after which PDGF-BB (100 ng/ml) or an equivalent volume of medium was added for 15 min. Crude membranes were prepared as described previously [11] and extracted for 20 min with NP 40 buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 10 U/ml aprotinin, 20 μM leupeptin, 100 μM Na^+ orthovanadate, 2.5 mM EDTA, 10 mM NaF). The crude membrane extracts were cleared by centrifugation at 4°C for 15 min, and immunoprecipitation was performed with an anti-PDGF β-type receptor serum [13] at a 1:250 dilution. The immunoprecipitates were collected by adding protein-A Sepharose, and were washed three times with NP40 buffer and two times with kinase buffer (20 mM Hepes, pH 7.5, 10 mM $MnCl_2$, 100 μM sodium orthovanadate). Kinase activity was assayed in 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP for 10 min at 25°C. Reactions were stopped by the addition of 50 μl sample buffer and boiled for 10 min. The proteins were separated by electrophoresis on a 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel and visualized by scanning with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Cell labelling with [^{32}P]orthophosphate. Confluent NRK and v-*src*-NRK cells in 90-mm dishes were placed in serum-free Dulbecco's modified Eagle's medium for 20 h. The cells were washed three times with phosphate-free medium and labelled with 500 μCi/ml [^{32}P]orthophosphate for 2 h at 37°C. The cells were then incubated with or without 100 ng/ml PDGF-BB for an additional 15 min before membrane extracts were prepared as described above. The protein contents of the membrane extracts were determined by the Bradford method [15] and the same amount of protein from each cell line was used for immunoprecipitation with anti-PDGF receptor antibody. The immunoprecipitates were analyzed on a 7.5% SDS–polyacrylamide gel and visualized by scanning with a phosphorimager.

Immunoblotting. Confluent cells were placed in serum-

free Dulbecco's modified Eagle's medium for 20 h. The membrane pellets were prepared as described above and the protein contents were determined by the Bradford assay [15]. The pellets were extracted with 100 μl sample buffer (95°C, 10 min) and centrifuged for 10 min at 15 000 × *g*. Equivalent amounts of the supernatant protein were electrophoresed on 7.5% SDS–polyacrylamide gels and electrophoretically transferred to an Immobilon poly(vinylidene difluoride) membrane (Millipore, Bedford, MA). The membrane was treated at room temperature with agitation for 1 h in blocking solution (PBS, 4% BSA, 0.05% Tween 20), and washed twice for 5 min each in wash solution (PBS, 0.1% BSA, 0.02% Tween 20). The anti-PDGF receptor β-type antibody was added at a 1:10 000 dilution in wash solution and incubated overnight at 4°C with agitation. The membrane was washed 3 times for 7 min each in wash solution and incubated with goat anti-rabbit IgG(H + L)-horseradish peroxidase conjugate (Bio-Rad laboratories, Hercules, CA) at a 1:10 000 dilution in wash solution for 1 h at room temperature with agitation. The membrane was agitated (3 × 20 min) in wash solution, and receptor was detected by enhanced chemiluminescence (Amersham, Bucks., UK) according to the manufacturer's instructions.

PolyA$^+$-mRNA isolation and Northern blot analysis. Cells were grown to confluence and placed in serum-free media overnight. Cells were homogenized in a solution containing 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 200 μg per ml proteinase K, and 0.5% SDS and incubated for 1 h at 37°C. mRNA was enriched by poly (A)$^+$ selection on oligodeoxythymidylate cellulose [16] and precipitated with sodium acetate and ethanol. Then, 17 μg of polyA$^+$-mRNA was denatured at 65°C in 2.2 M formaldehyde, 1 mM EDTA, 5 mM Na^+ acetate, 20 mM Mops (pH 7.0; RNA buffer) in 50% (v/v) formamide, fractionated electrophoretically on a 1% (w/v) agarose gel in RNA buffer, and transferred onto a nylon membrane (GeneScreen, Du Pont, Boston, MA) with 10 × SSC (1.5 M NaCl, 0.15 M Na^+ citrate). The membrane was prehybridized at 42°C in the presence of 50% formamide (deionized), 1% dextran sulfate and 100 μg per ml denatured salmon sperm DNA for 4 h. The same solution was used for hybridization (42°C, overnight) except that salmon sperm DNA was omitted. The ^{32}P-labelled probe, corresponding to the transmembrane, the juxtamembrane and the first tyrosine kinase segments of the human PDGF β-type receptor [17] was denatured and added at a concentration of $2 \cdot 10^6$ cpm per ml. The membrane was washed in 2 × SSC at room temperature for 10 min, then twice in 2 × SSC, 1% SDS at 60°C for 20 min, and finally visualized by scanning with a phosphorimager.

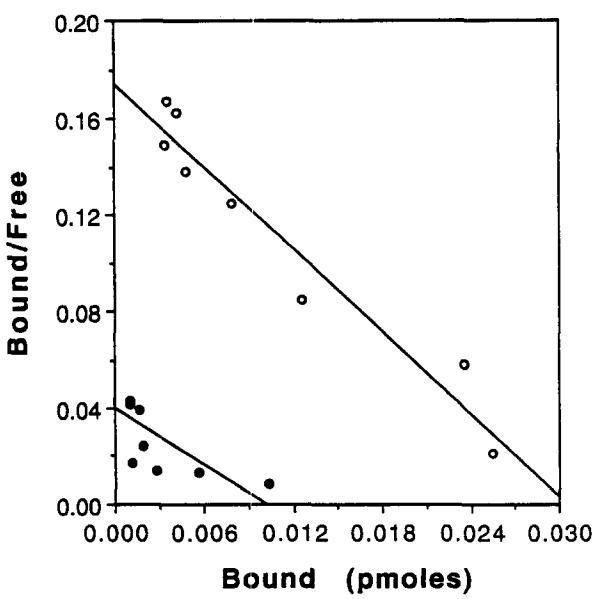

Fig. 1. Comparison of ^{125}I-PDGF binding to v-*src*-NRK and NRK cells. Binding of ^{125}I-PDGF-BB (28250 cpm, 25 fmol) to 10^6 v-*src*-NRK (●) or NRK (○) fibroblasts was measured in duplicate in the presence of increasing concentrations of unlabelled PDGF (3 pM–800 pM) as described in Section 2. Data were fitted with the program LIGAND [18].

Table 1
PDGF Receptor levels on transformed NRK cells

Cell line	Surface PDGF receptors/cell [a]	K_d (M) [a]
NRK	$2.2 \cdot 10^5$	$3.5 \cdot 10^{-9}$
pmt-NRK	$2.0 \cdot 10^5$	$4.2 \cdot 10^{-9}$
v-*src*-NRK	$2.9 \cdot 10^4$	$8.2 \cdot 10^{-9}$

[a] Calculated with the program LIGAND [18].

3. Results

3.1. Levels of PDGF receptor in transformed cells

We have reported previously that PDGF-dependent autophosphorylation of the PDGF receptor in v-*src*-transformed NRK fibroblasts is profoundly suppressed [11]. To investigate the cause of this suppression we first measured cell surface binding of ^{125}I-PDGF (Fig. 1, Table 1). The number of PDGF receptors on v-*src*-NRK cells was only 13% of the number on NRK cells, while the affinity of the residual receptors was reduced only 2-fold. In contrast, the number of PDGF receptors on pmt-NRK cells was 90% of the number on NRK cells. The reduction in the number of receptors on v-*src*-NRK cells was confirmed by Western blotting equal amounts of membrane protein from v-*src*-NRK, pmt-NRK and NRK cells with an anti-PDGF receptor antibody (Fig. 2). Levels of PDGF receptor in the v-*src*-NRK cells were only 10% of the levels in NRK or pmt-NRK cells. The activity of the residual PDGF receptors in an autophosphorylation assay in vitro was then tested in immunoprecipitates prepared from v-*src*-NRK cells with antibodies to the PDGF receptor (Fig. 3). The levels of PDGF receptor autophosphorylation in immuno-

precipitates from unstimulated and stimulated v-*src* NRK cells were only 40% and 25%, respectively, of the levels observed in unstimulated and stimulated NRK cells. When corrected for the 7- to 10-fold reduction in PDGF receptor numbers in v-*src*-NRK cells, the specific autokinase activity of the residual PDGF receptors was actually increased by v-*src* transformation. Moreover, the residual PDGF receptor in v-*src*-NRK cells still showed a 4.3-fold increase in autophosphorylation in response to PDGF, suggesting that the tyrosine kinase activity of the residual receptors could still be activated by PDGF binding. In contrast, levels of PDGF receptor autophosphorylation were similar in immunoprecipitates prepared from pmt-NRK and NRK cells.

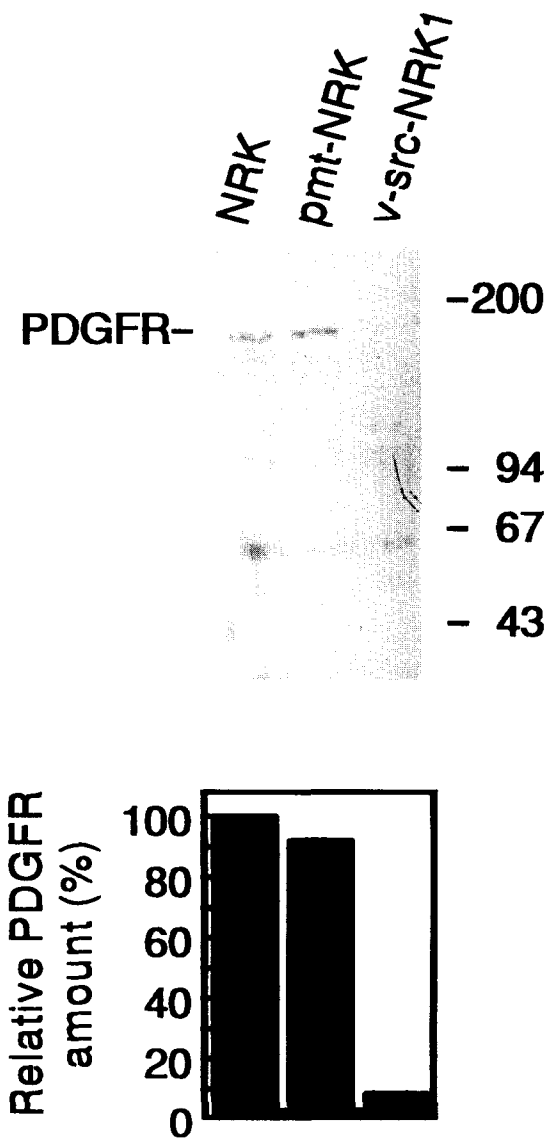

Fig. 2. Comparison of PDGF receptor levels in NRK, pmt-NRK, and v-*src*-NRK cells by Western blotting. Membrane protein (30 μg) from each cell line was electrophoresed on a 7.5% SDS–polyacrylamide gel and transferred to an Immobilon membrane. The membrane was incubated with rabbit anti-PDGF receptor (β-type) antiserum, washed and incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate. Bound antibody was detected by enhanced chemiluminescence, and quantitated with a phosphorimager. The sizes of molecular weight markers are given in kDa, and the position of the PDGF receptor is indicated.

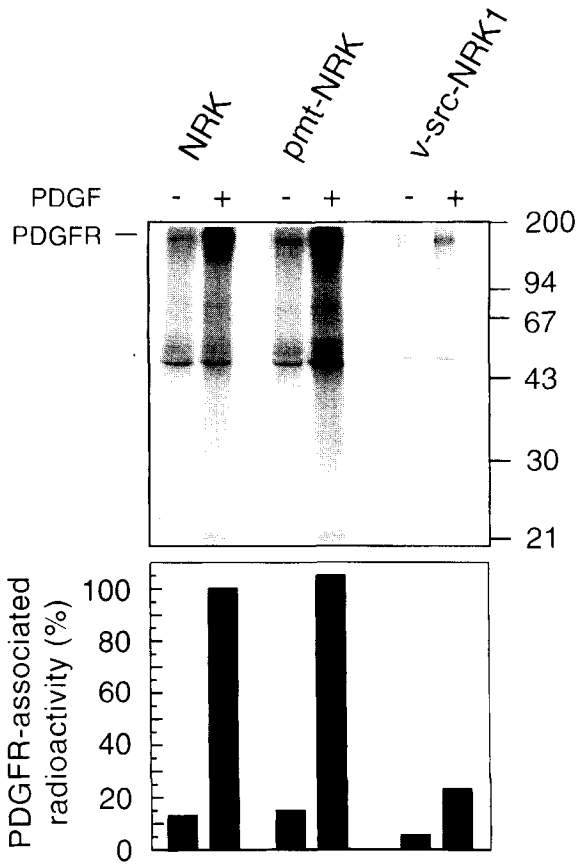

Fig. 3. Comparison of PDGF receptor kinase activity in NRK, pmt-NRK, and v-*src*-NRK cells. Confluent and serum starved cells were incubated with (+) or without (−) PDGF-BB (100 ng/ml) for 15 min at 37°C. Membranes were prepared and extracted with detergent, and equivalent amounts of protein were incubated with an anti-PDGF receptor (β-type) antiserum. The immunoprecipates were then autophosphorylated with 10 μCi γ-[^{32}P]ATP for 10 min at 25°C, and the phosphoproteins were separated by SDS–polyacrylamide gel electrophoresis and visualized and quantitated with a phosphorimager. The radioactivity associated with the PDGF receptor was expressed as a percentage of the value in stimulated NRK cells. These data were representative of 4 similar experiments.

3.2. Phosphorylation state of the PDGF receptor

To determine whether the reduction in PDGF receptor levels was a consequence of autocrine PDGF production, v-*src*-NRK and NRK cells were labelled to equilibrium with [^{32}P]orthophosphate and treated with PDGF or buffer only. The PDGF receptor was isolated by immunoprecipitation, and purified by electrophoresis on SDS–polyacrylamide gels which were analysed with a phosphorimager (Fig. 4). The observation that phosphorylation of the PDGF receptor increased 15-fold in v-*src*-NRK cells compared with a 6-fold increase in NRK cells clearly established that the PDGF receptor was not maximally phosphorylated in the transformed cells, and hence suggested that significant

amounts of PDGF were not secreted into the medium by the cells. In addition comparison of the levels of PDGF receptor phosphorylation in v-*src*-NRK cells with the levels in NRK cells before (16-fold lower) and after (7-fold lower) PDGF stimulation yielded results which were consistent with the 7-fold decrease in PDGF receptor levels observed in a direct binding assay (Table 1). There is thus no evidence that the level of phosphoamino acids in the unstimulated PDGF receptor is elevated in NRK fibroblasts following v-*src* transformation. This result is clearly different from Rat-1 fibroblasts transfected with the human EGF receptor where expression of the v-*src* oncogene resulted in a 2.5-fold increase in the level of phosphoamino acids in the unstimulated human EGF receptor [19].

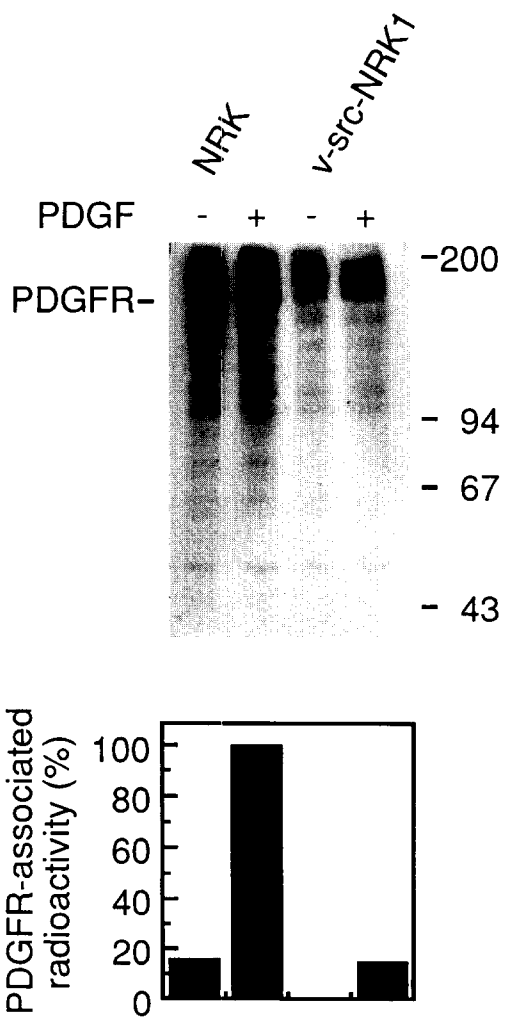

Fig. 4. The PDGF receptor is not fully phosphorylated in v-*src*-NRK cells. Confluent and serum-free cells were labelled with 0.5 mCi [^{32}P]orthophosphate per ml for 2 h at 37°C. The cells were then incubated with (+) or without (−) 100 ng/ml PDGF for 15 min. Membranes were prepared and extracted with detergent and equal amounts of membrane extract were immunoprecipitated with anti-PDGF receptor antiserum. The immunoprecipitates were analyzed by electrophoresis on 7.5% SDS–polyacrylamide gels, and the PDGF receptor was visualized and quantitated by scanning with a phosphorimager. The radioactivity associated with the PDGF receptor was expressed as a percentage of the value for stimulated NRK cells. This experiment was performed only once because of the high levels of radioactivity (4 mC_i) required.

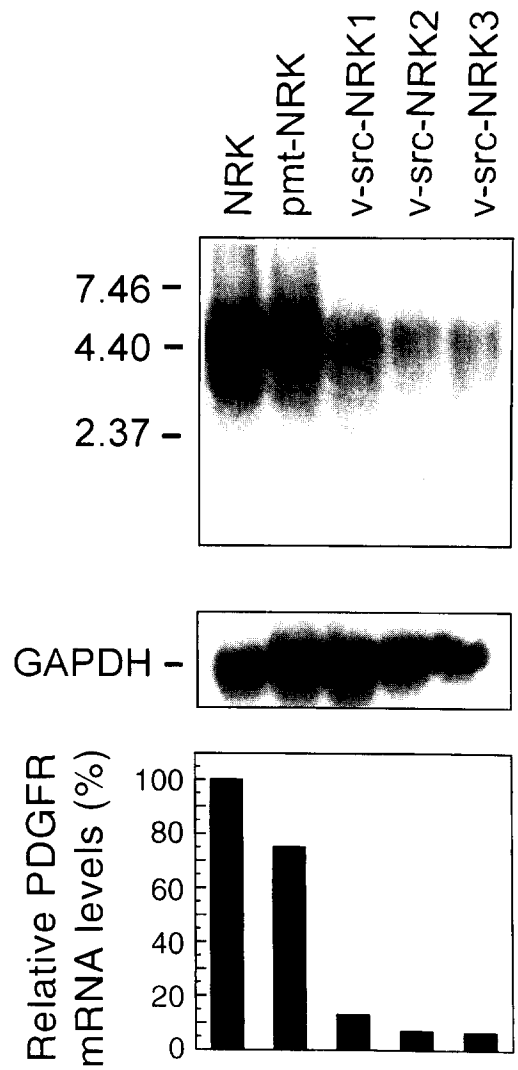

Fig. 5. Comparison of PDGF receptor mRNA levels in NRK, pmt-NRK, and v-*src*-NRK cells. PolyA$^+$-mRNA (17 μg) from NRK and pmt-NRK cells, and from three independent clones of v-*src*-transformed-NRK cells, was size fractionated on a 1% agarose gel and transferred onto a nylon membrane. The membrane was hybridized sequentially with [^{32}P]-labelled probes for the human β-type PDGF receptor and for GAPDH. The radioactive bands were visualized and quantitated by scanning with a phosphorimager, and the values obtained after normalization for the variation in levels of GAPDH mRNA were expressed as a percentage of the value obtained for NRK cells. Sizes of RNA standards are indicated in kb.

3.3. Levels of PDGF receptor mRNA

We next measured levels of PDGF receptor mRNA in v-*src*-NRK cells by Northern blotting. In three independently isolated clones of v-*src*-transformed NRK cells the amounts of PDGF receptor mRNA were between 6 and 13% of the amounts observed in the parent NRK cells (Fig. 5). In contrast, levels of PDGF mRNA in pmt-NRK

cells were 75% of the levels in NRK cells. The reduction in mRNA levels did not appear to be a consequence of autocrine PDGF production, since treatment of NRK fibroblasts with 100 ng/ml PDGF-BB for 20 h before mRNA isolation did not result in any reduction in the levels of PDGF receptor mRNA (data not shown).

4. Discussion

Although there is general agreement in the literature that the EGF receptor in v-*src*-transformed mouse and rat fibroblasts is down-regulated, the exact mechanism is controversial. Gray and Macara [6] reported that the number of EGF receptors was unchanged, but that their affinity for EGF was decreased 10-fold following v-*src* transformation. In contrast, Decker [4], and Weber and coworkers [5] have reported that the number of EGF binding sites in v-*src*-transformed rat fibroblasts is less than 10% of the number in the parent cells, and that this decrease is due to a decrease in EGF receptor protein [5], which in turn is due to a decrease in EGF receptor mRNA [3].

When we observed that PDGF-dependent autophosphorylation of the PDGF receptor was significantly reduced in NRK fibroblasts transformed with the v-*src* oncogene [11], we therefore measured the number and affinity of PDGF binding sites on the cell surface. The results clearly indicated that the number of PDGF binding sites was reduced following transformation, but that the affinity of the residual PDGF receptors was unchanged. No such decrease in PDGF receptor numbers was observed in NRK fibroblasts transformed by the pmt oncogene. Measurements of the levels of PDGF receptor protein directly by Western blotting or indirectly by immunoprecipitation and autophosphorylation were in complete agreement with the results of the binding studies. Northern blotting of poly-A^+ mRNA prepared from v-*src*-NRK, pmt-NRK and NRK cells with PDGF receptor probes revealed, in the v-*src*-NRK cells only, a decrease in PDGF receptor mRNA which closely paralleled the decrease in PDGF receptor protein. Furthermore a similar reduction in PDGF receptor mRNA levels was observed with three independently isolated clones of v-*src*-transformed NRK cells, indicating that the reduction was not caused by a spontaneous mutation occurring after the transformation event. We conclude that transformation of NRK fibroblasts by the v-*src* oncogene results in a reduction in the levels of PDGF receptor mRNA, which ultimately leads to a reduction in the PDGF binding sites at the cell surface.

Although we have not directly measured PDGF levels in media conditioned by v-*src*-NRK cells, two observations suggest that the reduction in cell surface PDGF receptors is not caused by increased receptor internalization and degradation following binding of autocrine PDGF. Firstly treatment of untransformed NRK fibroblasts with 100 ng/ml PDGF-BB for 20 h did not alter PDGF receptor mRNA levels. This observation is consistent with the data of Heldin and co-workers who found that following treatment of untransformed human foreskin fibroblasts with 100 ng/ml PDGF-BB the levels of PDGF receptor mRNA actually increased 4-fold at 8 h and returned to normal at 24 h, while levels of cell surface PDGF binding decreased 3-fold by 1 h and remained at that level for 24 h [20]. Hence the observed 7-fold reduction in PDGF receptor mRNA levels following v-*src* transformation is not consistent with the effects of autocrine PDGF production predicted from the effects observed with exogenous PDGF. Secondly, when v-*src*-NRK cells which had been labelled with [^{32}P]orthophosphate were stimulated with PDGF, the fold increase in PDGF receptor autophosphorylation was greater than in untransformed NRK cells (Fig. 4). If any autocrine PDGF had been present the basal level of PDGF receptor autophosphorylation should have been increased, and hence the fold increase observed following addition of PDGF would have been expected to be reduced in the transformed cells. We conclude that production of autocrine PDGF by v-*src*-transformed NRK cells does not contribute significantly to the observed reduction in cell surface PDGF receptors.

Paradoxically the reduction in cell surface PDGF receptors is probably not the cause of the cells' failure to respond mitogenically to PDGF [11]. Our results clearly show that the transformed cells still possess an average of 29 000 binding sites each, and that the ability of these residual receptors to autophosphorylate in response to PDGF is undiminished. Moreover, Racker and co-workers have previously demonstrated that phospholipase C-mediated phosphoinositide hydrolysis in v-*src*-transformed mouse 3T3 cells is still activated by PDGF [10]. A more likely explanation than the reduction in PDGF binding sites for the lack of a mitogenic response is that the cells are proliferating at a maximal rate because the v-*src* protein stimulates a component in the signalling cascade downstream from the PDGF receptor.

The effects of v-*src* transformation on the PDGF receptor are clearly different from the effects of transformation by the v-*ras* oncogene or by the middle T antigen of polyoma virus. Mouse 3T3 fibroblasts transformed with the v-*ras* oncogene have undiminished levels of PDGF receptor [8–10]. However, the autophosphorylating ability of the PDGF receptor is substantially reduced by an inhibitory factor present in the membranes of the v-*ras*-transformed cells [8], and there is no activation of phospholipase C-mediated phosphoinositide hydrolysis [10] or induction of c-*myc* and c-*fos* expression in response to PDGF [9]. In the case of pmt-NRK cells, levels of PDGF binding are only slightly lower than in NRK cells (Table 1), the PDGF receptor is still autophosphorylated in response to PDGF, and a mitogenic response to PDGF is still observed [11]. The differences between pmt-NRK and v-*src*-NRK cells are particularly surprising because pmt transformation is thought to be a consequence of the

activation of c-*src* following binding of the middle T antigen [21] and hence imply that the signal transduction pathways form c-*src* and v-*src* are not identical. In conclusion transformation of NRK fibroblasts by the v-*src* oncogene differs from transformation by the v-*ras* or pmt oncogenes in that levels of PDGF receptor mRNA are reduced with a consequent decrease in the numbers of PDGF receptors on the cell surface.

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